ELSEVIER

Biochimica et Biophysica Acta 1452 (1999) 133–144

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.elsevier.com/locate/bba

Particles binding β_2-integrins mediate intracellular production of oxidative metabolites in human neutrophils independently of phagocytosis

Lena Serrander [a,*], Jenny Larsson [b], Helen Lundqvist [b], Maria Lindmark [a], Maria Fällman [c], Claes Dahlgren [d], Olle Stendahl [a]

[a] *Department of Medical Microbiology, University Hospital of Linköping, S-58185 Linköping, Sweden*
[b] *Department of Pathology II, Linköping University, Linköping, Sweden*
[c] *Department of Medical Microbiology, University of Umeå, Umeå, Sweden*
[d] *Department of Medical Microbiology, University of Göteborg, Göteborg, Sweden*

Received 3 April 1998; received in revised form 3 August 1999; accepted 27 August 1999

Abstract

Complement-opsonised particles are readily ingested by human neutrophils through a complement receptor-mediated process leading to phagolysosome fusion and production of oxidative metabolites. To investigate the complement receptor 3 (CR3)-associated signal system involved, cells were challenged with protein A-positive, heat-killed *Staphylococcus aureus* to which antibodies with specificity for the subunits of the β_2-integrins, i.e. anti-CD11b (the α subunit of CR3) and anti-CD18 (the β subunit of CR3), were bound through their Fc moiety. Despite not being ingested by the neutrophils, the surface associated anti-CD18- and anti-CD11b-coated particles were able to activate the neutrophil NADPH-oxidase. Also anti-CD11a- (the α subunit of LFA-1) and to a lesser extent anti-CD11c- (the α subunit of CR4) coated particles were able to trigger the NADPH-oxidase. The NADPH-oxidase was activated without extracellular release of reactive oxygen species. The activity was inhibited by cytochalasin B, suggesting a necessary role for the cytoskeleton in the signalling pathway that activates the oxidase. We show that particle-mediated cross-linking of β_2-integrins on the neutrophil surface initiates a signalling cascade, involving cytoskeletal rearrangements, leading to an activation of the NADPH-oxidase without phagosome formation or extracellular release of reactive oxygen species.

Keywords: Neutrophil; Integrin; PANSORBIN; Signalling; Cytoskeleton; NADPH-oxidase

1. Introduction

Neutrophils play a key role in our cellular inflammatory response to pathogens. During the inflammatory response, neutrophils are readily mobilised to the site of inflammation where they phagocytose pathogens or fragments of damaged tissue [1]. Subsequently they release a variety of proteolytic enzymes and produce oxygen species that destroy the engulfed material and may also damage host tissue in the vicinity of the inflamed site [2]. During activation, neutrophils increase their oxygen consumption unrelated to mitochondrial respiration. Oxygen is used as an electron acceptor, the electrons being ferried across the membrane to oxygen by a unique enzyme, the phagocytic NADPH-oxidase. The reduced oxygen species formed, i.e. superoxide and

* Corresponding author.

0167-4889/99/$ – see front matter
PII: S0167-4889(99)00123-8

hydrogen peroxides, can efficiently kill microorganisms when combined with the neutrophil granule enzymes. The NADPH-oxidase is a highly regulated enzyme complex that is composed of a number of different proteins [3]. In resting cells, the NADPH-oxidase is dormant and its protein components are separated into cytosolic and membrane compartments [4]. During assembly of the functional oxidase, the cytosolic proteins translocate to the membrane, a process that is initiated at the cell surface upon sensing of an inflammatory mediator or recognition of a prey. The active NADPH-oxidase has long been considered a plasma membrane-bound system, but all membrane components necessary for superoxide production are present also in the specific granule membrane [5]. In fact, unperturbed cells store 80–85% of the membrane-associated oxidase components in their granules [6]. Most of the oxygen free radicals formed during phagocytosis of complement-opsonised yeast are indeed retained intracellularly indicating that the oxidase is assembled primarily in the phagosome/phagolysosome membrane [7,8].

A prerequisite for engulfment of a microbe and a subsequent activation of the oxidase is recognition of the prey. Binding of microorganisms to neutrophils occurs either through bacterial lectins that bind directly to carbohydrate-containing phagocyte receptors or through recognition of opsonins, a recognition mediated by specific receptors for the Fc part of immunoglobulins (FcγR II and IIIB) and receptors recognising cleavage products from complement factor 3 (CR 1 and 3). Complement receptor 3 (CR3; CD11b/CD18) is a member of the integrin family of adhesion molecules and recognises the surface-bound opsonin C3bi, collagen, zymosan, fibrinogen as well as ICAM molecules exposed on the endothelium of the blood vessel wall [9]. This molecule is composed of two protein chains. Neutrophils expose three forms of the β_2-integrin subfamily, i.e. CR3 (CD11b/CD18), LFA-1 (CD11a/CD18), CR4 or p150/95 (CD11c/CD18), which all have a common β-chain but differ with respect to the structure of the α-chain [10]. CR3, with an α-chain of 165 kDa (CD11b), is the predominant integrin present on neutrophils. Binding of a complement-opsonised microorganism to CR3 initiates signalling events that ultimately lead to engulfment of the prey. The ingestion phase is followed by mobilisation of the neutrophil granules to form a phagolysosome, the organelle in which the killing arsenal of the phagocytes is activated. Stimulation of neutrophils via CR3 leads to phosphorylation of a number of proteins, activation of different phospholipases, a rise in intracellular free calcium concentration and actin polymerisation [11–14]. The precise signalling pathways regulating neutrophil functions such as adhesion, pseudopod formation, engulfment of the prey and phagolysosome formation are yet to be identified. This is true also with respect to the assembly and activation of the NADPH-oxidase. Both the α (CD11b) and the β (CD18) subunits of CR3 are integral membrane proteins that may signal through their cytoplasmic tails. The signalling pathway leading to NADPH-oxidase activity is currently believed to involve tyrosine phosphorylation events through *src*-like kinases [15], the activation of small GTP-binding proteins [16] and activation of phospholipase D (PLD) [17]. Although activation of PLD precedes phagocytosis and is not directly linked to any reorganisation of cytoskeletal proteins [18], the enzyme has been implicated in activating the NADPH-oxidase [19–21].

The aim of the present study was to investigate the β_2-integrin signalling in relation to activation of the NADPH-oxidase. We used the antibody-based system, in which antibodies linked to protein A-positive particles (PANSORBINS) are allowed to interact with the CR3 subunits of the neutrophil β_2-integrins. We found that the signals generated during occupation of CR3 and the other β_2-integrins activate the NADPH-oxidase without any phagosome formation, degranulation or extracellular release of reactive oxygen species.

2. Material and methods

2.1. Chemicals

Luminol (5-amino-2,3-dihydro-1,4-phthalazindione), formylmethionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA), cytochalasin B (CB), *p*-hydroxyphenylacetate (PHPA) and nitro blue tetrazolium (NBT) were obtained from Sigma (St. Louis, MO, USA) and PANSORBINS from Calbiochem (San Diego, CA, USA). Dextran and Ficoll-Hypaque were obtained from Pharmacia

Fine Chemicals (Uppsala, Sweden). Carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK) was a gift from Dr. S. Schlegel (University Hospital of Geneva, Switzerland). Cytochrome *c* (cyt *c*), horseradish peroxidase (HRP), superoxide dismutase (SOD) and catalase (CAT) was purchased from Boehringer-Mannheim (Germany). Luminol was dissolved in 0.1 M NaOH to 20 mM. Dimethyl sulphoxide (DMSO) was used to dilute fMLP and PMA to 10 mM.

2.2. Antibodies

Mouse monoclonal anti-CD18 antibodies (IB4, subclass IgG_{2b}) originated from Dr. S. Wright (Rockefeller University, New York, USA). The monoclonal antibodies M/Gl, subclass IgG_{2a} (anti-CD11b), BL Leuk 11a, subclass IgG_1 (anti-CD11a) and BL-4H4, subclass IgG_1 (anti-CD11c) were purchased from Monosan via Bio-Zac AB (Järfälla, Sweden). OKM1 subclass IgG_{2b} (anti-CD11b) was bought from Ortho Diagnostic Systems (Sollentuna, Sweden). The antibody CBR-p150/4G1, subclass IgG_{2a} (antiCD11c) was bought from Bender Med-Systems (Vienna, Austria) and clone 38, subclass IgG_{2a} (anti-CD11a) from Ancell (Bayport, USA). Rabbit-anti-mouse (RAM) and mouse negative control IgG_{2b}, clone DAK-GO5, was purchased from Dakopatts (Glostrup, Denmark).

2.3. Neutrophil preparation

Heparinised blood was collected from blood donors and separated according to Bøyum [22]. After dextran sedimentation, brief hypotonic lysis and Ficoll-Hypaque centrifugation, the cells were washed twice, resuspended to 1×10^7/ml in Krebs-Ringer phosphate buffer supplemented with 10 mM glucose, 1.2 mM Mg^{2+} and 1 mM Ca^{2+}, pH 7.3 (KRG) and kept on melting ice and used within 2–4 h. All further dilutions were made in KRG.

2.4. HL60 cell preparation

HL60 cells were grown in RPMI 1640, supplemented with 10% heat-inactivated foetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Differentiation was induced by growing the cells for 4–7 days in the presence of 1.3% DMSO. The cells were then postmitotic and responded well to fMLP and PMA with NADPH activity. Cells were washed twice, resuspended in KRG to 1×10^7/ml and kept on ice before stimulation.

2.5. Preparation of antibody-coated PANSORBINS

PANSORBINS (specially hardened and heat-killed protein A-positive *Staphylococcus aureus*, Cowan 1 strain) were coated with antibodies directed against either the α- or the β-chain of CR3. The PANSORBIN particles bind antibodies in an oriented fashion; the Fc part of the molecules will stick to protein A present on the particles, leaving the antigen-binding moieties of the antibodies exposed and free to bind to the β_2-integrin molecules on the neutrophil surface. Antibodies directed against CD18, CD11a, CD11b, CD11c or control mouse IgG_{2b} were used to coat the particles, which were then added to the neutrophils.

One volume of the PANSORBIN solution (10% w/v) was centrifuged and the pellet was resuspended to the original volume in PBS (pH 7.3) supplemented with the antibodies (0.2 mg/ml of anti-CD11a, -CD11b, -CD11c and -CD18 respectively). During binding of IgG_1 antibodies to the PANSORBINS, the NaCl concentration was raised to 3 M and pH adjusted to 9.0. The mixtures were shaken continuously for 1 h at ambient temperature. The particles were then washed twice and resuspended in 0.2 M sodium borate buffer (pH 9.0) and the process of chemical coupling the Fc part of the antibodies to the PANSORBINS was achieved by the addition of 20 mM dimethylpimelimidate. The coupling step was terminated after 30 min by washing once and followed by incubation for 2 h in 0.2 M ethanolamine (pH 8.0). The antibody-coated particles were finally washed and resuspended to 10^{10}/ml in PBS containing 0.01% merthiolate [23].

The particles were presented at a ratio of 100 particles/neutrophil in all experiments.

2.6. Phagocytosis and binding assay

Neutrophils were preincubated at 37°C for 5 min before FITC-labelled particles were presented at a ratio of 100:1. After 20 min at 37°C, an equal

volume of crystal violet (0.5 mg in 0.15 M NaCl) was added to quench extracellular FITC-labelled particles [16]. Phagocytosis and binding was determined for 50 cells/sample in a fluorescence microscope. Phagocytic index is shown as fluorescent cells/total PMN and binding is shown as particles bound/total PMN.

2.7. Chemiluminescence measurements

Chemiluminescence (CL) was measured in a 6-channel Biolumat LB9505 (Berthold, Wildbad, Germany) using disposable 4 ml polypropylene tubes with a 1 ml reaction mixture. One million cells in 0.9 ml were allowed to equilibrate for 5 min at 37 C in KRG before 0.1 ml stimuli was added. Final concentrations of reactants were 100 nM fMLP, 100 nM PMA, 20 μM luminol, 10^8/ml PANSORBINS, 5 μg/ml CB and 10 μM zLYCK.

Two different reaction mixtures were prepared for measuring intra- and extracellular CL respectively [24]. Reaction mixtures for measuring extracellularly generated CL contained 1 mM azide and 4 U/ml HRP. Azide was added to inhibit MPO-dependent intracellular CL. Tubes used for measuring intracellularly generated CL contained SOD (200 U/ml) and CAT (2000 U/ml) to scavenge extracellularly released superoxide anion and hydrogen peroxide.

HRP (4 U/ml) was added to all experiments with HL60 cells since the amount of myeloperoxidase released from these cells is so low that the extracellular release of oxygen radicals otherwise could be underestimated using a peroxidase-dependent assay [25]. Peak values of the CL curves were chosen to express oxidative metabolism. The kinetic figures shown are representative for each set of experiments.

2.8. Superoxide anion generation

The increase in absorbency seen during reduction of ferricytochrome *c* was followed spectrophotometrically at 550 nm in a Beckman spectrophotometer. Each sample contained 1.5 mg cytochrome *c*, 2×10^6 PMNs in a final volume of 1 ml in KRG. The reference cuvette also contained 200 U of SOD. Anti-CD18 PANSORBINS were added after 5 min and O_2^- generation was recorded continuously for 30 min at 37°C. [26]

2.9. Hydrogen peroxide production

Adherent neutrophils (10^6) were challenged with anti-CD18 PANSORBINS (10^8) for 45 min. Each sample contained 8 U HRP and 0.5 mg PHPA in KRG (final volume of 1 ml). The increase in $(PHPA)_2$ fluorescence was recorded in a LS50 (Perkin Elmer) with an excitation and emission wavelength of 317 nm and 400 nm respectively [26,27].

Azide, 1 mM final concentration, was added to some samples to block the H_2O_2 consuming enzymes MPO and catalase and by that allow measurement of the total amount of H_2O_2 produced. H_2O_2 is a membrane-traversing molecule and the intracellularly produced H_2O_2 is rapidly consumed by catalase and MPO, i.e. without azide, only extracellularly released H_2O_2 can be detected. Intracellularly produced H_2O_2 was calculated by subtracting H_2O_2, measured without azide, from H_2O_2 measured in the presence of azide [28,29].

2.10. NBT test

Neutrophils were incubated at a concentration of 10^6/ml at 37°C for 20 min with 10^8/ml anti-CD18 antibody-coated PANSORBINS and 1 mg/ml NBT, which is water-soluble and weakly yellow under physiological conditions. NBT is reduced by the metabolites produced by the oxidase and forms a blue precipitate, formazan. Cells turning blue were considered positive.

2.11. Statistics

At least three experiments were performed under each condition. Non-paired Student's *t*-test was used to evaluate differences in between populations. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Activation of the neutrophil NADPH-oxidase during interaction with PANSORBINS exposing antibody-binding sites specific for CD18 and CD11

When neutrophils were challenged with PANSOR-

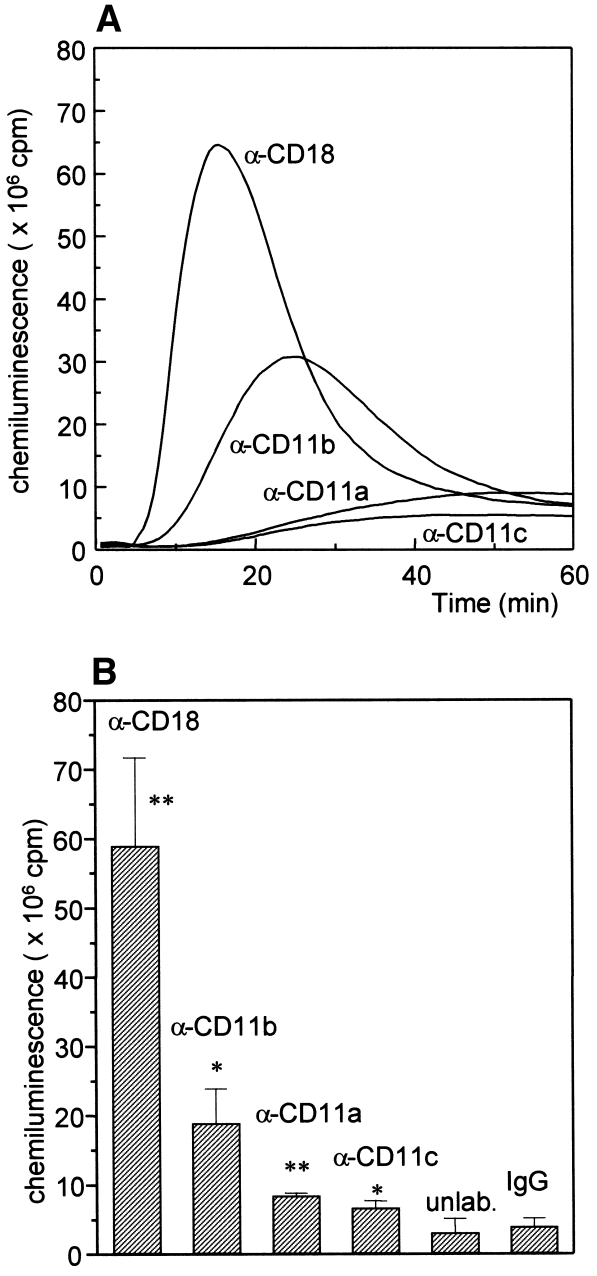


Fig. 1. NADPH-oxidase activity in neutrophils challenged with PANSORBINS coated with antibodies to β_2-integrin subunits. (A) A representative chemiluminescence measurement with four different antibody-coated PANSORBINS. (B) Peak amplitudes of each antibody-coated particle type. Data are shown as mean ± S.E.M. ($n = 3$–26). Statistically significant differences compared to unlabelled particles are marked ($^*P < 0.05$, $^{**}P < 0.01$).

BINS exposing antibodies directed against CD18, the β-chain of CR3, the neutrophil NADPH-oxidase was activated and generated reactive oxygen species measured as a chemiluminescence response (Fig. 1A). This was also true when antibodies directed against CD11b (M/Gl, subclass IgG_{2a} or OKM1, subclass IgG_{2b}), the α-chain of CR3, were used to coat the PANSORBINS (Fig. 1A). A smaller oxidative response was induced when particles exposing anti-CD11a (BL Leuk 11a, subclass IgG_1)- or anti-CD11c (BL-4H4, subclass IgG_1)-specific antibodies were used to challenge the phagocytes (Fig. 1B). When subclass-matched anti-CD11a (clone 38, subclass IgG_{2a}) and anti-CD11c (CBR-p150/4G1, subclass IgG_{2a}) antibodies were used, the oxidative response was more pronounced (65 and 35% of the response induced by IB4, data not shown). NBT test was performed to correlate the oxidative responses to particle interaction. More than 50% neutrophils had bound but not ingested particles and only neutrophils with bound particles contained formazan deposits excluding a soluble activating factor (data not shown).

Anti-CD18 antibodies (10 μg/ml) in solution did not activate the neutrophil NADPH-oxidase, not even when these antibodies were cross-linked with a secondary antibody (rabbit-anti-mouse, 30 μg/ml) after 60 min at 37°C (data not shown).

3.2. *Interaction of PANSORBINS exposing antibodies specific for CD18 and CD11 with neutrophils*

Complement-opsonised particles are readily phagocytosed by neutrophils and it is assumed that the products, formed by the NADPH-oxidase during phagocytosis, are released primarily into the phagolysosome. To localise the particles a fluorescent quenching technique was used [7]. Very few fluorescent PANSORBIN particles could be detected intracellularly after addition of crystal violet whereas many were observed bound. Despite the fact that PANSORBINS exposing Fab moieties specific for either the α- or β-chain of CR3 activate the oxidase, these particles bind to the neutrophils, but are not ingested ([18], Fig. 2A).

Whereas neutrophils challenged with serum-opsonised PANSORBINS show a phagocytic index (phag-

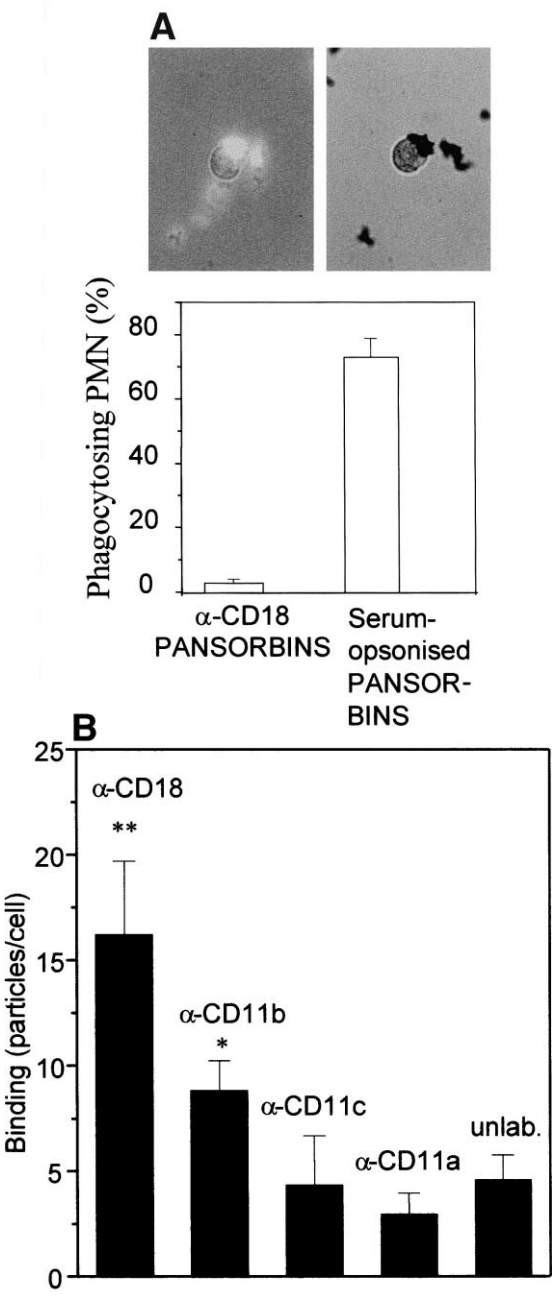
ocytosing PMN/total number of PMN) of 73 ± 6%, neutrophils challenged with anti-CD18 PANSORBINS (100 particles/PMN) display a phagocytic index of 2.9 ± 1.33% (Fig. 2A). Ingestion of antibody-coated PANSORBINS could not be increased when neutrophils were prestimulated with 5–50 nM PMA or fMLP or when PANSORBINS were labelled with higher concentrations (2 mg/ml) of antibodies.

The binding was the highest for the anti-CD18 PANSORBINS followed by particles coated with anti-CD11b (M/Gl, subclass IgG_{2a} or OKM1, subclass IgG_{2b}), anti-CD11c (BL-4H4, subclass IgG_1), and anti-CD11a (BL Leuk 11a, subclass IgG_1) (Fig. 2B). Binding correlated to the peak values of the different antibodies coating the PANSORBINS with a correlation coefficient of 0.97.

3.3. *Localisation of the NADPH-oxidase activity induced by PANSORBINS exposing anti-CD18 antibodies*

Activation of NADPH-oxidase by soluble ligands like the chemoattractant fMLP causes an extracellular response whereas phagocytic receptors (CR3) normally induce intracellular activity probably originating from the phagosome. This led us to investigate whether the reactive oxygen species induced by PANSORBINS are produced inside or released extracellularly. Luminol is a small membrane-penetrating molecule that can be used to monitor extracellular release of reactive oxygen species as well as generation of oxygen radicals in intracellular compartments [24,26]. The chemiluminescence response induced during interaction between neutrophils and anti-CD18 PANSORBINS was virtually insensitive to scavengers of extracellularly released O_2^- (SOD) and H_2O_2 (catalase), the two main metabolites generated by the neutrophil NADPH-oxidase, whereas azide inhibited the response; thus the response was mainly intracellular (Fig. 3A,B).

We could confirm the results using the cytochrome



→

Fig. 2. Anti-CD18 PANSORBINS are bound but not ingested to human neutrophils. (A) The micrographs show neutrophils and anti-CD18 PANSORBINS before (left) and after (right) addition of crystal violet which leaves the ingested particles fluorescent and quenches extracellular particles. (The neutrophil is in focus in order to detect ingested particles, 100× oil immersion fluorescence objective.) The bar graph show the phagocytic index (phagocytosing neutrophils/total number of neutrophils) for neutrophils interacting with anti-CD18 antibody-labelled PANSORBINS or complement-opsonised PANSORBINS (25% normal human serum for 30 min at 37°C) respectively (100 particles/neutrophil). (B) The graph shows the amount of binding for each type of particle/neutrophil. Binding of particles coated with anti-CD18 and anti-CD11b were significantly different from binding of unlabelled particles (*$P < 0.05$ or **$P < 0.01$). Data are shown as mean ± S.E.M. ($n = 3$–5).

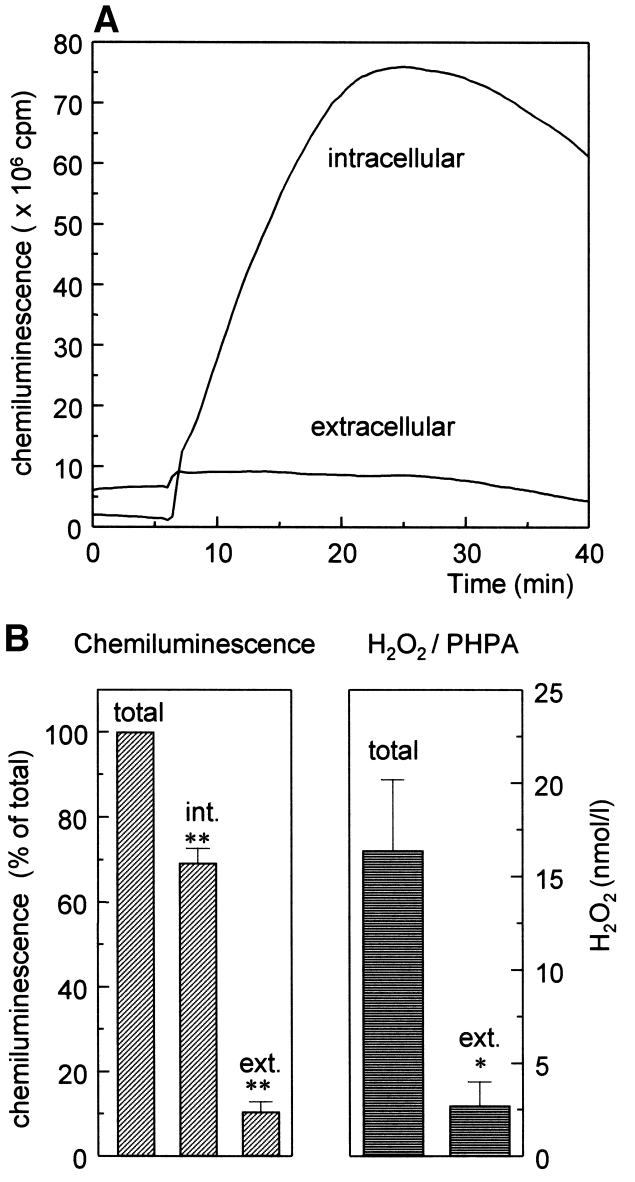


Fig. 3. Localisation of the oxidase activity in neutrophils induced by anti-CD18 coated particles. (A) Representative chemiluminescence curves with 200 U/ml SOD and 2000 U/ml catalase in the mixture to measure intracellular activity and 4 U/ml HRP and 1 mM azide in the mixture to measure extracellular activity. (B) The left panel shows mean of peak values ± S.E.M. from three to five experiments. The right panel shows H_2O_2 measurements using the fluorescent probe PHPA. Total H_2O_2 production was measured in the presence of 1 mM azide whereas the extracellular production was measured in the absence of azide. The mean H_2O_2 production from 10^6 cells after 45 min is shown ± S.E.M. ($n=3$). Statistically significant differences compared to total production is shown by $^*P<0.05$ or $^{**}P<0.01$.

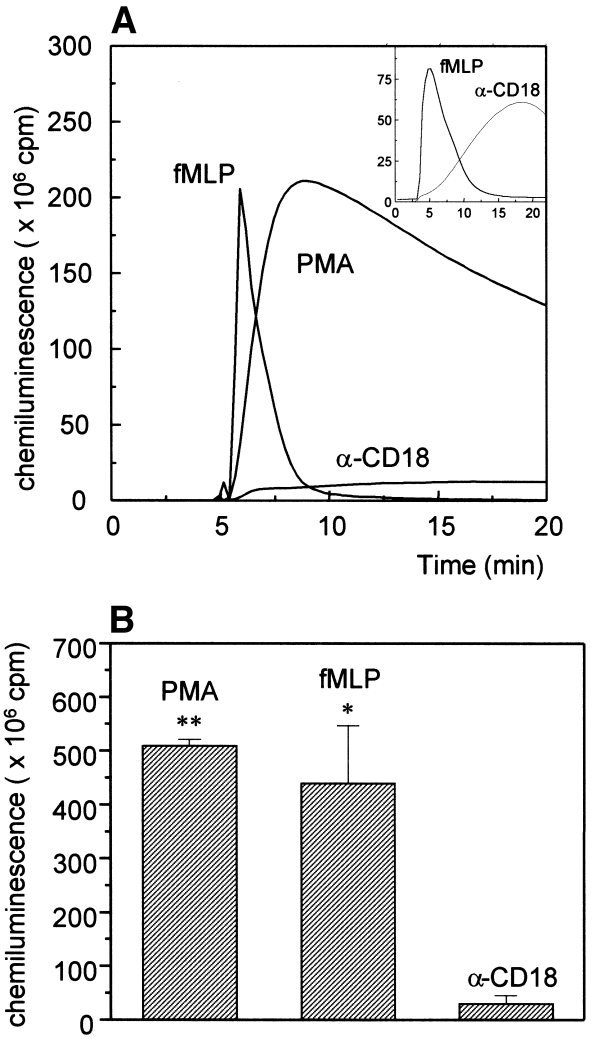


Fig. 4. Chemiluminescence response in HL60 cells exposed to anti-CD18 PANSORBINS, fMLP or PMA. (A) Representative measurement for HL60 cells stimulated with 100 nM fMLP, 100 nM PMA and 10^8/ml anti-CD18 PANSORBINS. The inset in A shows a representative measurement of the relative proportions for fMLP and anti-CD18 PANSORBINS, for comparison, in neutrophils. (B) Peak values for four experiments as mean peak amplitude ± S.E.M. for the same stimuli as above. Statistically significant differences compared to anti-CD18 PANSORBINS are shown by $^*P<0.05$ or $^{**}P<0.01$.

c reduction technique to measure extracellular release (data not shown) and the HRP-PHPA technique (Fig. 3B), that is, the reactive oxygen species formed during neutrophil interaction with PANSORBINS are not released.

To further confirm the intracellular NADPH-oxidase activity induced by anti-CD18 PANSORBINS, we used the promyelotic cell line HL60. DMSO-dif-

ferentiated HL60 cells express CR3 on the surface, and these cells are also able to assemble the different NADPH-oxidase components into a functional electron transporting complex. They lack, however, secondary granules and thereby the intracellular pool of the membrane-bound part of the NADPH-oxidase, i.e. they cannot generate intracellular chemiluminescence [25]. Reactive oxygen species were produced when we challenged differentiated HL60 cells with PMA of fMLP, but no production could be detected when these cells were challenged with anti-CD18-coated PANSORBINS (Fig. 4). Neutrophils challenged with fMLP and anti-CD18-coated PANSORBINS are shown in the inset in Fig. 4 for comparison, showing that the oxidase activity induced by anti-CD18 PANSORBIN stimulation is comparable to the activity induced by fMLP in neutrophils.

3.4. The role of F-actin polymerisation and PLD activation for the neutrophil response induced during interaction with PANSORBINS

How is CR3-induced activation relayed to the intracellular NADPH? To evaluate the role of cytoskeleton-receptor communication in CR3-mediated respiratory burst activity, we studied the effect of cytochalasin B. Cytochalasin B shortens actin filaments by binding to the fast growing barbed ends, blocking the addition of new actin monomers. As a consequence, signalling may be changed as seen in fMLP-stimulated neutrophils where cytochalasin B enhances the release of oxygen species. We found that the oxidase activity induced by anti-CD18-coated PANSORBINS was largely inhibited ($85 \pm 5\%$; mean ± S.E.M.) in the presence of cytochalasin B (5 μg/ml) (Fig. 5).

Previous studies on different signalling pathways during phagocytosis of complement-opsonised yeast have pointed to the importance of PLD in the signalling events during particle engulfment [12]. Other reports have also suggested PLD activation as an important signal for receptor-mediated O_2^- production using zLYCK, a protease inhibitor which inhibits the activation of PLD rather than the enzyme itself [30–32]. The fact that the anti-CD18 PANSORBINS activate the neutrophil NADPH-oxidase independently of phagocytosis offered an opportunity to pursue the

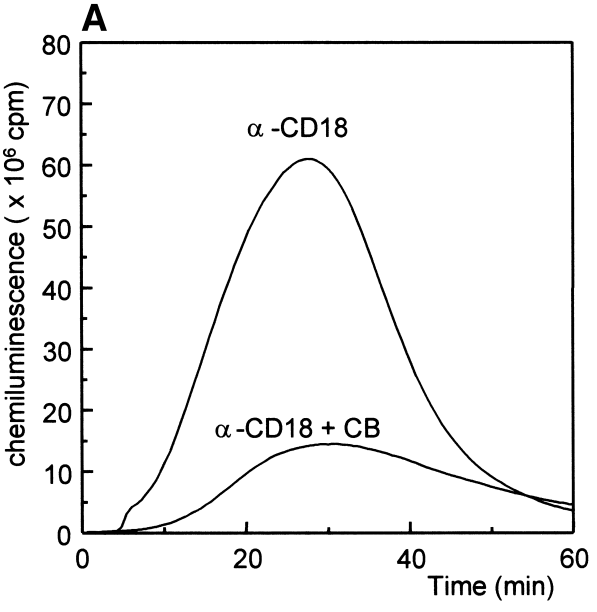


Fig. 5. The effect of cytochalasin B on the chemiluminescence response in neutrophils stimulated with anti-CD18 PANSORBINS. Cytochalasin B (5 μg/ml) was added 5 min prior to stimulation with anti-CD18 PANSORBINS and chemiluminescence was recorded for 60 min. The figure shows a representative curve out of three experiments.

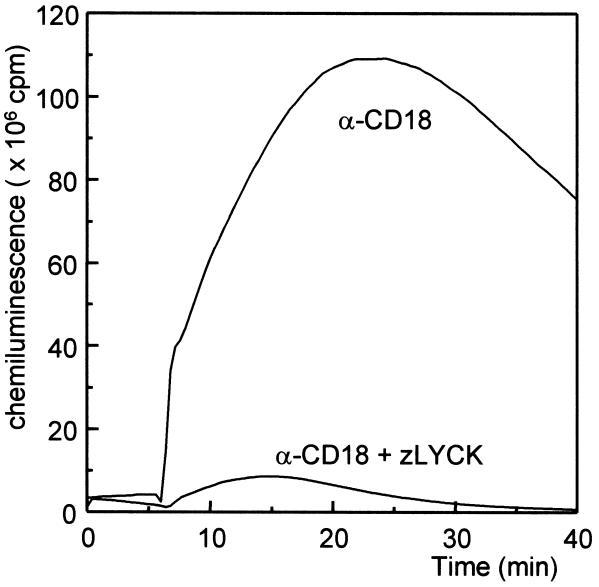


Fig. 6. The effect of zLYCK on NADPH activity induced by anti-CD18 particles in human neutrophils. zLYCK (10 μM), was added 5 min before anti-CD18 PANSORBINS were added to the neutrophils and chemiluminescence was measured continuously for 40 min. The figure shows a representative curve out of five experiments.

question whether PLD activation is involved in CD18-mediated oxidase activation independently of phagocytosis.

PLD is indeed activated when neutrophils are challenged with anti-CD18 PANSORBINS and 10 μM zLYCK inhibits the PLD activity by 60% [18]. Under the same conditions the respiratory burst was inhibited by 87 ± 7%, i.e. to background levels (mean ± S.E.M., $n = 5$) (Fig. 6). Higher concentrations did not inhibit PLD further (data not shown). These results support the hypothesis that the signalling pathway from CR3 to production of reactive oxygen species is linked to activation of PLD, but that the remaining 40% of PLD activity are not sufficient to generate oxidase activity.

This might either suggest that PLD has to be fully activated for oxidase activity or that the upstream factor inhibited by zLYCK is a more important signalling molecule inducing oxidative metabolism.

4. Discussion

Our data clearly show that binding of a particle to the α subunit for CR3 as well as to the common β subunit of the β_2-integrins activates the oxidase. It has been claimed that occupation of CR3 in neutrophils does not necessarily lead to an activation of the oxidase [33,34]. Earlier studies using opsonised yeast particles have the drawback that other structures, besides C3bi, potentially capable of binding to the neutrophil may be present on complement-opsonised particles [7,12]. To overcome this problem, we have used particles coated with specific antibodies against integrins to show that the β_2-integrin CR3 indeed mediates an intracellular production of free oxygen species independently of phagocytosis. This way both specificity and a particular stimuli can be achieved, i.e. clustering of the receptors. It was also evident that local clustering of receptors is a prerequisite for oxidase activation, since neither antibodies on their own nor cross-linking is sufficient to generate an oxidative response. The quantitative differences between the responses induced by the PANSORBINS coated with anti-CD18 and anti-CD11 antibodies respectively, most probably relates to the difference in the number of particles bound to the phagocytes. Microscopic examination and quantification showed that more anti-CD18 PANSORBINS than anti-CD11 PANSORBINS were associated with the neutrophils.

It is intriguing that particles coated with antibodies to phagocytic receptors bind and activate the cells, but do not mediate phagocytosis. The reason they are not ingested might be that (i) other surface molecules need to be activated, (ii) a special epitope needs be bound, or, maybe more likely, (iii) the density or affinity of the ligand is not sufficient to induce conformational changes and uptake. There are data demonstrating that CD18, transfected into non-professional phagocytes, in itself can mediate uptake of yeast particles supplying support for the latter [35].

The technique of stimulating receptors with monoclonal antibodies coupled to particles or surfaces has also been used by Berton et al. [33], showing that anti-CD18 PANSORBINS, as well as anti-CD11a and c, but not particles bearing anti-CD11b, activate the oxidase upon binding to the neutrophils. Contradictory to these results, we show that anti-CD11b PANSORBINS (as well as anti-CD18-coated particles) bind and activate the neutrophils more avidly than particles exposing anti-CD11a or c. These discrepancies could be due to the choice of antibodies and method to determine NADPH-oxidase activity. Using other clones of CD11a and c antibodies, it was clear that also these integrins are able to transduce signals mediating oxidase activity. We did also test the anti-CD11b antibody (OKM1) used by Berton et al. [33] with similar results as with the anti-CD11b shown in Fig. 1.

In most studies, the oxidase activation has been measured with the cytochrome *c* reduction assay or similar methods to detect O_2^-/H_2O_2 [11,33,34]. In general these systems measure extracellular release of reactive oxygen species. CL measurements or measuring H_2O_2 with PHPA in the presence of azide makes it possible to detect intracellular oxidase activity. H_2O_2 can cross both organelle and plasma membrane and azide blocks both MPO and catalase that both consume H_2O_2. Once these enzymes are blocked, the whole production of H_2O_2 can be detected extracellularly. The results obtained with the PHPA system correlate well with the results obtained by CL, confirming that the oxygen metabolites formed during neutrophil interaction with PANSORBINS take place at an intracellular site.

The luminol-amplified chemiluminescence reaction in neutrophil has two main characteristics: a peroxidase is required as well as reduced oxygen species derived from the NADPH-oxidase. It can be postulated that in order to obtain an intracellular chemiluminescence response, the neutrophil peroxidase (MPO) and the oxygen radical generating system (the NADPH-oxidase) have to be present in the same subcellular compartment. A phagolysosome formed through fusion between the plasma membrane-derived phagosome, azurophil granules containing MPO and specific granules containing the cytochrome *b* of the oxidase, fulfils the criteria for an intracellular organelle in which the chemiluminescence reaction can take place. We have here observed, however, that PANSORBINS interacting with neutrophils through CR3 can activate the generation of reactive oxygen species intracellularly, without formation of a phagolysosome. An intracellular production of reactive oxygen species without formation of a phagolysosome is achieved also when cells are challenged with a calcium-specific ionophore [36] and the PKC activator PMA [26]. The intracellular compartment containing the assembled oxidase after stimulation with ionomycin, PMA or anti-CD18 PANSORBINS has not yet been identified, though there was a recent report demonstrating that oxidase activity could take place in a compartment positive for alkaline phosphatase [37]. One hypothesis is that the antibody-coated particles induce macro-endocytosis and the endosomes later undergo granule fusion similarly as phagosomes. Some support for this hypothesis comes from our experiments with HL60 cells. These cells are devoid of specific granules, where the main reservoir of cytochrome *b* resides, and despite the fact that these cells are able to generate superoxide as well as hydrogen peroxide extracellularly when stimulated with PMA or the chemoattractant fMLP, no intracellular activity can be detected with any stimulus [25]. Accordingly, differentiated HL60 cells did not produce any detectable CL when challenged with anti-CD18 PANSORBINS.

The function of the intracellularly generated reactive oxygen species is still unresolved. Recent evidence from both plant and animal cells suggests that these metabolites may act as intracellular messengers, mediating activation of transcription factors and protein phosphorylation events [38,39]. Whether the intracellularly produced oxygen metabolites in response to β_2-integrin stimulation are part of a signal transduction cascade, leading to functional effects in the cell, is an intriguing question that warrants further studies. The inhibitory effect of cytochalasin B on CL induced by anti-CD18 PANSORBINS shows that a reorganisation of the cytoskeletal network is required for relaying a signalling cascade through CD18, even when particle uptake does not occur as a result of receptor binding. Data from Tapper et al. [40] suggest that the reorganisation of the cytoskeleton is important not for the signalling per se, but for clustering of the Fc receptors to enable a sufficient binding for signalling.

There is a link between PLD activation and CR3-mediated events. Fällman et al. [12] showed that the major part of diacylglycerol production leading to PKC activation during phagocytosis is mediated through PLD and we have earlier shown that inhibition of PLD activation by zLYCK leads to diminished phagocytosis [18].

The present demonstration that inhibition of PLD by zLYCK leads to complete inhibition of CD18-mediated oxidative activation suggests that CR3 signalling involves PLD activation. The fact that PLD activity is inhibited by 60% whereas the NADPH-oxidase activity is inhibited by almost 90% indicates that zLYCK has several targets and it cannot be ruled out that the protease-inhibiting effect of zLYCK could affect several proteases required for cell activation. Whereas the inhibition of fMLP-induced oxidative metabolism correlates closer to the inhibition of fMLP-induced PLD, using zLYCK, it is more likely that zLYCK inhibits one or more proteases involved closer in fMLP-induced activation than CR3-induced oxidative metabolism [30,31]. Also inhibition of complement-induced PLD activity and phagocytosis seems to correlate closer distinguishing two or more activation pathways for different functions induced by the same receptors [18]. The involvement of PLD in the activation of intracellular oxidase activity requires further studies.

Our present concept is that neutrophils have at least two different pathways to activate the oxidative response: one signalling pathway leading to oxidase activity which is highly dependent on functional cytoskeleton leading to activation of the intracellular

pool of the NADPH-oxidase and another mediating extracellular response which does not require an intact cytoskeleton. Here we have shown that phagocytic stimuli can induce intracellular production of oxidative metabolites without being ingested, suggesting some kind of granule-granule fusion. Further investigations are necessary to clarify the origin and function of this separately regulated intracellular oxidative activation.

Acknowledgements

We thank associate professor Tommy Sundquist for valuable discussions. This study was supported by grants from the Swedish Med. Res. Council (5968, 8298, 11222), King Gustav V 80 Year Foundation and Swedish Soc. against Rheumatism.

References

[1] E.J. Brown, Phagocytosis BioEssays 17 (1995) 109–117.
[2] H. Jaeschke, C.W. Smith, Mechanisms of neutrophil-induced parenchymal cell injury, J. Leukocyte Biol. 61 (1997) 647–653.
[3] L.M. Henderson, J.B. Chappell, NADPH oxidase of neutrophils, Biochim. Biophys. Acta 1273 (1996) 87–107.
[4] B. Dewald, M. Baggiolini, J.T. Curnutte, B.M. Babior, Subcellular localization of the superoxide-forming enzyme in human neutrophils, J. Clin. Invest. 63 (1979) 21–29.
[5] M.T. Quinn, M.L. Mullen, A.J. Jesaitis, J.G. Linner, Subcellular distribution of the Rap1A protein in human neutrophils: colocalization and cotranslocation with cytochrome b559, Blood 79 (1992) 1563–1573.
[6] O.W. Bjerrum, N. Borregaard, Dual granule localization of the dormant NADPH oxidase and cytochrome b559 in human neutrophils, Eur. J. Haematol. 43 (1989) 67–77.
[7] J. Hed, O. Stendahl, Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils, Immunology 45 (1982) 727–736.
[8] A. Johansson, A.J. Jesaitis, H. Lundqvist, K.E. Magnusson, C. Sjölin, A. Karlsson, C. Dahlgren, Different subcellular localization of cytochrome b and the dormant NADPH-oxidase in neutrophils and macrophages: effect on the production of reactive oxygen species during phagocytosis, Cell Immunol. 161 (1995) 61–71.
[9] E.J. Brown, I.L. Graham, Macrophage and inflammatory cell matrix receptors: LFA-1, MAC-1, p150, 95 family, in: J.A. McDonald, R.P. Mecham (Eds.), Macrophage and Inflammatory Cell Matrix Receptors, Academic Press, San Diego, CA, 1991, pp. 39–79.
[10] M.A. Arnaout, Structure and function of the leukocyte adhesion molecules CD11/CD18, Blood 75 (1990) 1037–1050.
[11] B.V. Della, M. Grzeskowiak, D. Lissandrini, F. Rossi, Source and role of diacylglycerol formed during phagocytosis of opsonized yeast particles and associated respiratory burst in human neutrophils, Biochem. Biophys. Res. Commun. 177 (1991) 948–955.
[12] M. Fällman, M. Gullberg, C. Hellberg, T. Andersson, Complement receptor-mediated phagocytosis is associated with accumulation of phosphatidylcholine-derived diglyceride in human neutrophils. Involvement of phospholipase D and direct evidence for a positive feedback signal of protein kinase C, J. Biol. Chem. 267 (1992) 2656–2663.
[13] B. Walzog, R. Seifert, A. Zakrzewicz, P. Gaehtgens, K. Ley, Cross-linking of CD18 in human neutrophils induces an increase of intracellular free Ca^{2+}, exocytosis of azurophilic granules, quantitative up-regulation of CD18, shedding of L-selectin, and actin polymerization, J. Leukocyte Biol. 56 (1994) 625–635.
[14] M. Fällman, R. Andersson, T. Andersson, Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles, J. Immunol. 151 (1993) 330–338.
[15] G. Berton, L. Fumagalli, C. Laudanna, C. Sorio, Beta 2 integrin-dependent protein tyrosine phosphorylation and activation of the FGR protein tyrosine kinase in human neutrophils, J. Cell Biol. 126 (1994) 1111–1121.
[16] M.T. Quinn, Low-molecular-weight GTP-binding proteins and leukocyte signal transduction, J. Leukocyte Biol. 58 (1995) 263–276.
[17] S. Cockcroft, G.M.H. Thomas, A. Fensome, B. Geny, E. Cunningham, I. Gout, I. Hiles, N.F. Totty, O. Truong, J.J. Hsuan, Phospholipase D: A downstream effector of ARF in granulocytes, Science 263 (1994) 523–526.
[18] L. Serrander, M. Fällman, O. Stendahl, Activation of phospholipase D is an early event in integrin-mediated signalling leading to phagocytosis in human neutrophils, Inflammation 20 (1996) 439–450.
[19] D.E. Agwu, L.C. McPhail, S. Sozzani, D.A. Bass, C.E. McCall, Phosphatidic acid as a second messenger in human polymorphonuclear leukocytes. Effects on activation of NADPH oxidase, J. Clin. Invest. 88 (1991) 531–539.
[20] D. English, Involvement of phosphatidic acid, phosphatidate phosphohydrolase, and inositide-specific phospholipase D in neutrophil stimulus-response pathways, J. Lab. Clin. Med. 120 (1992) 520–526.
[21] D. Qualliotine-Mann, D.E. Agwu, M.D. Ellenburg, C.E. McCall, L.C. McPhail, Phosphatidic acid and diacylglycerol synergize in a cell-free system for activation of NADPH oxidase from human neutrophils, J. Biol. Chem. 268 (1993) 23843–23849.
[22] A. Bøyum, Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g, Scand. J. Clin. Lab. Invest. Suppl. 97 (1968) 77-89.

[23] E. Harlow, D. Lane, Preparing protein A bead-antibody affinity columns – direct coupling, in: E. Harlow, D. Lane (Eds.), Antibodies – a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988, pp. 521–525.
[24] C. Dahlgren, P. Follin, A. Johansson, R. Lock, H. Lundqvist, Å. Walan, Chemiluminescence as a means of following the function of phagocytic cells, Trends Photochem. Photobiol. 2 (1991) 427–443.
[25] C. Dahlgren, The calcium ionophore ionomycin can prime, but not activate, the reactive oxygen generating system in differentiated HL-60 cells, J. Leukocyte Biol. 46 (1989) 15–24.
[26] H. Lundqvist, P. Follin, L. Khalfan, C. Dahlgren, Phorbol myristate acetate-induced NADPH oxidase activity in human neutrophils: only half the story has been told, J. Leukocyte Biol. 59 (1996) 270–279.
[27] P.A. Hyslop, L.A. Sklar, A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes, Anal. Biochem. 141 (1984) 280–286.
[28] R.K. Root, J. Metcalf, N. Oshino, B. Chance, H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors, J. Clin. Invest. 55 (1975) 945–955.
[29] R.K. Root, J.A. Metcalf, H_2O_2 release from human granulocytes during phagocytosis. Relationship to release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H_2O_2: studies with normal and cytochalasin B-treated cells, J. Clin. Invest. 60 (1977) 1266–1279.
[30] A. Gervaix, G.C.R. Kessels, S. Suter, D.P. Lew, A.J. Verhoeven, The chymotrypsin inhibitor carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK) interferes with the neutrophil respiratory burst mediated by a signaling pathway independent of $PtdInsP_2$ breakdown and cytosolic free calcium, J. Immunol. 147 (1991) 1912–1919.
[31] G.C. Kessels, A. Gervaix, D.P. Lew, A.J. Verhoeven, The chymotrypsin inhibitor carbobenzyloxy-leucine-tyrosine-chloromethylketone interferes with phospholipase D activation induced by formyl-methionyl-leucyl-phenylalanine in human neutrophils, J. Biol. Chem. 266 (1991) 15870–15875.
[32] S.E. Schlegel-Haueter, T. Dudez, C. Ruef, S. Suter, in: Astra-Symposium on Phagocytes, Copenhagen, 19–20 August 1993 (Abstr.).
[33] G. Berton, C. Laudanna, C. Sorio, F. Rossi, Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils, J. Cell Biol. 116 (1992) 1007–1017.
[34] S.D. Wright, S.C. Silverstein, Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes, J. Exp. Med. 158 (1983) 2016–2023.
[35] J.C. Krauss, H. Poo, W. Xue, L. Mayo-Bond, R.F.W. Todd, H.R. Petty, Reconstitution of antibody-dependent phagocytosis in fibroblasts expressing fc gamma receptor iiib and the complement receptor type 3, J. Immunol. 153 (1994) 1769–1777.
[36] C. Dahlgren, A. Johansson, H. Lundqvist, O.W. Bjerrum, N. Borregaard, Activation of the oxygen-radical-generating system in granules of intact human neutrophils by a calcium ionophore (ionomycin), Biochim. Biophys. Acta 1137 (1992) 182–188.
[37] T. Kobayashi, J.M. Robinson, H. Seguchi, Identification of intracellular sites of superoxide production in stimulated neutrophils, J. Cell Sci. 111 (1998) 81–91.
[38] J. Nath, Y. Ohno, J.I. Gallin, D.G. Wright, A novel post-translational incorporation of tyrosine into multiple proteins in activated human neutrophils. Correlation with phagocytosis and activation of the NADPH oxidase-mediated respiratory burst, J. Immunol. 149 (1992) 3360–3371.
[39] J.H. Brumell, A.L. Burkhardt, J.B. Bolen, S. Grinstein, Endogenous reactive oxygen intermediates activate tyrosine kinases in human neutrophils, J. Biol. Chem. 271 (1996) 1455–1461.
[40] H. Tapper, S. Grinstein, Fc receptor-triggered insertion of secretory granules into the plasma membrane of human neutrophils – Selective retrieval during phagocytosis, J. Immunol. 159 (1997) 409–418.